Biochemical Pharmacology, 1966, Vol. 15, pp. 333–341. Pergamon Press Ltd., Printed in Great Britain.

# EFFECT OF NORETHANDROLONE, ACETOHEXAMIDE, AND ENOVID ON α-NAPHTHYLISOTHIOCYANATE-INDUCED HYPERBILIRUBINEMIA AND CHOLESTASIS*

ROBERT J. ROBERTS and GABRIEL L. PLAA

Department of Pharmacology, College of Medicine, The University of Iowa, Iowa City, Iowa, U.S.A.

(*Received* 21 *September* 1965; *accepted* 19 *October*)

**Abstract**—Potentiation of α-naphthylisothiocyanate (ANIT)-induced hyperbilirubinemia and cholestasis was demonstrated in mice after pretreatment with norethandolone, acetohexamide, or Enovid. Studies involving the components of Enovid showed that the ANIT-potentiating activity resided in the progestational component, norethynodrel, and not mestranol. Serum glutamic-pyruvic transaminase levels after norethandrolone, acetohexamide, or Enovid pretreatment were elevated in accordance with the degree of potentiation of ANIT. In the absence of ANIT, norethandrolone, acetohexamide, or Enovid treatment alone had no significant effect on any of the parameters studied. Additional studies on hexobarbital sleeping time and metabolism showed that norethandrolone and Enovid, but not acetohexamide, significantly shortened sleeping time and increased metabolism of hexobarbital *in vivo*. The potentiation of ANIT may be due to enhanced biotransformation of ANIT to an active metabolite. However, it is noted that numerous drugs associated with cholestasis in man have been found to stimulate microsomal enzyme systems. It is suggested that these agents and ANIT may also affect bilirubin secretion into bile. Until the mechanisms involved are resolved, definite conclusions cannot be made regarding the application of ANIT-potentiation as a laboratory tool for detecting cholestatic drugs.

INCREASING numbers of therapeutic agents have been associated with various forms of hepatic injury, the majority causing a cholestatic response.[1–4] The inability to detect the cholestatic potential of the majority of such drugs in the laboratory animal has prompted the following investigation with the chemical α-naphthylisothiocyanate (ANIT).

ANIT has been known for some time to cause a reproducible hyperbilirubinemic and cholestatic response in mice and rats.[5–7] Recent work in our laboratory has shown that ANIT-induced hyperbilirubinemia and cholestasis can be potentiated in mice by pretreatment with chlorpromazine.[8] The fact that chlorpromazine has been known to cause jaundice in humans prompted us to investigate the feasibility of using ANIT as a tool to demonstrate in the laboratory animal the clinical manifestations of such potential cholestatic drugs. The rationale behind these studies has been that, although the cholestatic properties of these drugs may be too weak to elicit hyperbilirubinemia and/or cholestasis in experimental animals when administered alone, these properties might be manifested when these agents are administered to animals in combination with a threshold dose of a cholestatic chemical like ANIT. The purpose of this paper is to report findings of such work done with drugs implicated with cholestasis.

* This work was supported by funds from the United States Public Health Service (Research Grant AM-08083 and Training Grant 5T1-GM-141). A preliminary report was presented before the American Society for Pharmacology and Experimental Therapeutics in August 1965.

## MATERIALS AND METHODS

Male Swiss-Webster mice, obtained from Arthur Sutter Farms (25–35 g) and maintained unrestricted on laboratory diet and tap water, were randomized 10 per cage and used 5 to 9 days after arrival. All compounds were administered orally in a 1% carboxymethylcellulose (CMC) suspension to deliver the proper dosage in 0·01 ml/g body weight; CMC (0·01 ml/g body wt.) was administered as a control such that all experimental groups received an equal number of treatments. The drug treatments included norethandrolone, acetohexamide, and Enovid. In addition, preliminary investigations were conducted with norethynodrel, mestranol, methyltestosterone, chlorpropamide, and erythromycin estolate.

*Analytical methods.* In the bilirubin studies mice were treated at selected time intervals with the various agents, prior to ANIT administration; 24 hr after ANIT administration the mice were sacrificed by cardiac puncture under ether anesthesia, The individual blood samples were collected in Kahn tubes which had previously. been coated with 0·1 ml of 1·6% sodium oxalate solution and dried. The blood samples were centrifuged at 3000 rpm for 20 min, after which a 0·2-ml sample of plasma from each animal was analyzed colorimetrically for total bilirubin, by the method of Ferro and Ham.[9] A Bausch and Lomb Spectronic 20 spectrophotometer in conjunction with cuvettes and micro-space adaptors (Aloe Scientific, St. Louis) was used to determine quantitatively the absorbance of the samples.

An indirect method of determining the presence or absence of bile flow by using intravenously administered fluorescein, as described by Plaa and Becker,[10] was used in the cholestasis experiments. Fluorescein (20 mg/kg) was dissolved in isotonic $NaHCO_3$ and injected into the tail vein 15 min before examination for fluorescence. At the time of sacrifice the bile duct and gall bladder were exposed and visualized under u.v. light. The absence of fluorescence in the bile duct was defined as cholestasis.

Whole-body levels of hexobarbital were determined by the method of Axelrod *et al.*,[11] following the suggestions of Fouts (personal communication). These modifications consisted of substituting NaCl-saturated 0·5 M citrate buffer, pH 5·5, for phosphate buffer, pH 5·5; substituting heptane containing 1·5% isoamylalcohol for petroleum ether; and reading the absorbance of the final 0·8 M phosphate buffer (pH 11) extract at both 280 mμ and 245 mμ. The original whole-body homogenate was prepared by homogenizing the animal in a Waring blender for 20 sec with 2 ml of 1·15% KCl solution per g body weight. The homogenate was strained through cheesecloth and the final volume adjusted to 100 ml with distilled water. A 5-ml aliquot of the final homogenate was run through the hexobarbital analytical procedure.

Serum glutamic-pyruvic transaminase (SGPT) levels were determined by the method of Reitman and Frankel,[12] with materials obtained from Dade Reagents, Inc. (Miami, Fla.).

*Acute and chronic hyperbilirubinemia experiments*

Mice were given norethandrolone (20 mg/kg) or acetohexamide (50 mg/kg) 24 and 12 hr prior to ANIT (80 mg/kg) administration. In the acute Enovid experiments a dose of 50 mg/kg was given 24 hrs before ANIT, while in the chronic study with

Enovid, 2 mg/kg was given once daily for 5 days before ANIT administration. The components of Enovid, mestranol and norethynodrel, were assessed for hyperbilirubinemic potentiation activity by the administration of equivalent amounts (norethynodrel 49·25 mg/kg, mestranol 0·75 mg/kg) 24 hr prior to ANIT administration.

*Cholestasis experiments*

Norethandrolone, acetohexamide, Enovid, and ANIT were given in the same manner and dosages as in the hyperbilirubinemia experiments. However, the animals were examined for cholestasis 10 hr after ANIT administration rather than at the 24-hr interval used in the hyperbilirubinemia studies. After 10 hr, only about 20% of the animals treated with ANIT alone exhibited bile stasis. Therefore, the 10-hr interval enabled potentiation of ANIT-induced cholestasis to be more easily determined.

*Hexobarbital sleeping times and total body levels of hexobarbital*

Since the possibility arose that the agents tested might also affect liver microsomal activity, studies were initiated to assess this property indirectly. Hexobarbital sleeping times and whole-body levels were determined after pretreatment with norethandrolone, acetohexamide, or Enovid in a manner similar to the hyperbilirubinemia experiments. In these experiments, however, hexobarbital was substituted for the ANIT treatment. Sleeping times were run after a dosage of hexobarbital, 125 mg/kg, i.p. The sleeping times began with the loss of the righting reflex and terminated with the regaining of it. Following the same pretreatment procedure, total-body hexobarbital levels were determined 90 min after a dose of hexobarbital 200 mg/kg.

*SGPT determinations*

Treatment with norethandrolone, acetohexamide, Enovid, and ANIT were identical with those in the hyperbilirubinemia experiments. SGPT levels were determined 24 hr after the administration of ANIT.

*Statistical evaluation of the data*

The statistical methods used are described by Steel and Torrie.[13] The analysis used in the hyperbilirubinemia experiments was Duncan's new multiple-range test. The cholestatic data were compared by the chi-square method with the Yates correction. The total-body hexobarbital levels were compared by the Student's *t* test. The level of significance used in all determinations was P equal to or less than 0·05. In the tables, for the parametric data, mean values that are not significantly different ($P > 0{\cdot}05$) are connected by the same significance line; mean values that are significantly different ($P < 0{\cdot}05$) are not connected by the same significance line.

## RESULTS

Norethandrolone, an anabolic steroid which has been associated with cholestasis,[14] given in a dose of 20 mg/kg 24 and 12 hr before ANIT administration, caused a significant potentiation of the ANIT-induced hyperbilirubinemia (Table 1). Attempts at showing a linear dose–response relationship with norethandrolone were not successful, although significant potentiation of ANIT-induced hyperbilirubinemia was obtained with a dose of norethandrolone as low as 2·5 mg/kg. Norethandrolone itself had no significant effect on plasma bilirubin levels.

TABLE. 1 POTENTIATION OF ANIT-INDUCED HYPERBILIRUBINEMIA AFTER NORETHANDROLONE ADMINISTRATION

| Treatment* (N = 10) | Plasma bilirubin conc. (mg/100 ml)† | Significance (P < 0·05)‡ |
|---|---|---|
| Norethandrolone-ANIT | 4·9 ± 0·6 | \| |
| ANIT | 1·1 ± 0·3 | \| |
| Norethandrolone | 0·3 ± 0·1 | \| |
| Control | 0·3 ± 0·1 | \| |

* Norethandrolone (20 mg/kg, p.o.) was given 24 and 12 hr prior to ANIT administration (80 mg/kg, p.o.). N is the number of animals per treatment group, throughout the tables.

† Total plasma bilirubin levels are expressed as mg/100 ml of plasma (mean ± S.E.) Plasma bilirubin levels were determined 24 hr after ANIT administration (Tables 1–4).

‡ Throughout the tables, mean values which are not significantly different (P > 0·05) are connected by the same significance line; mean values which are significantly different (P < 0·05) are not connected by the same significance line.

TABLE 2. POTENTIATION OF ANIT-INDUCED HYPERBILIRUBINEMIA AFTER ACETOHEXAMIDE ADMINISTRATION

| Treatment* (N = 20) | Plasma bilirubin conc. (mg/100 ml) | Significance (P < 0·05) |
|---|---|---|
| Acetohexamide–ANIT | 1·8 ± 0·3 | \| |
| ANIT | 0·9 ± 0·2 | \| |
| Acetohexamide | 0·3 ± 0·1 | \| |
| Control | 0·3 ± 0·1 | \| |

* Acetohexamide (50 mg/kg, p.o.) was given 24 and 12 hr prior to ANIT administration (80 mg/kg, p.o.).

TABLE 3. POTENTIATION OF ANIT-INDUCED HYPERBILIRUBINEMIA AFTER ACUTE AND CHRONIC PRETREATMENTS WITH ENOVID

| Acute treatment* (N = 10) | Plasma bilirubin conc. (mg/100 ml) | Significance (P < 0·05) |
|---|---|---|
| Enovid–ANIT | 2·4 ± 0·3 | \| |
| ANIT | 1·1 ± 0·3 | \| |
| Enovid | 0·2 ± 0·1 | \| |
| Control | 0·2 ± 0·1 | \| |
| Chronic treatment† (N = 10) | | |
| Enovid–ANIT | 1·6 ± 0·3 | \| |
| ANIT | 0·9 ± 0·3 | \| |
| Enovid | 0·5 ± 0·1 | \| |
| Control | 0·3 ± 0·1 | \| |

* Acute treatment with Enovid consisted of a single dose, 50 mg/kg, p.o., 24 hr prior to ANIT administration (80 mg/kg, p.o.).

† Chronic treatment with Enovid consisted of 2 mg/kg, p.o., given once daily for five days prior to ANIT administration (80 mg/kg, p.o.).

Acetohexamide, an oral hypoglycemic agent which has been associated with drug-induced cholestasis,[15] also caused a significant potentiation of the ANIT-induced hyperbilirubinemia (Table 2). Acetohexamide iteslf had no significant effect on plasma bilirubin levels.

Several articles appearing in the literature recently[16–18] concerning the development of jaundice after the use of oral contraceptives prompted us to try the following series of experiments with Enovid. In the acute experiment shown in Table 3, a single dose of Enovid, 50 mg/kg significantly potentiated the ANIT-induced hyperbilirubinemia. As with norethandrolone, we were unable to show a linear dose–response association with acute Enovid treatment, although significant potentiation of ANIT-induced hyperbilirubinemia was obtained with a single dose of Enovid (12·5 mg/kg). Chronic Enovid treatment with doses of 2 mg/kg also resulted in approximately a twofold increase in the hyperbilirubinemia response to ANIT. Neither acute nor chronic Enovid treatment itself has any effect on plasma bilirubin levels.

In order to determine the relative involvement of the two components of Enovid in the potentiation of ANIT, mestranol and norethynodrel were administered singly in addition to being given in combination. The results of this experiment are shown in Table 4.

TABLE 4. EFFECT OF ENOVID AND ITS COMPONENTS ON ANIT-INDUCED HYPERBILIRUBINEMIA

| Treatment* (N = 10) | Plasma bilirubin conc. (mg/100 ml) | Significance (P < 0·05) |
|---|---|---|
| Enovid–ANIT | 1·8 ± 0·3 | |
| Mestranol–norethynodrel–ANIT | 1·8 ± 0·4 | |
| Norethynodrel–ANIT | 1·7 ± 0·5 | |
| ANIT | 0·7 ± 0·1 | |
| Mestranol–ANIT | 0·6 ± 0·1 | |

* All compounds were given once, 24 hr prior to ANIT (80 mg/kg, p.o.) administration. Enovid was given in a dose of 50 mg/kg, p.o., while a comparable dose of norethynodrel (49·25 mg/kg, p.o.) and mestranol (0·75 mg/kg, p.o.) was given.

Norethynodrel–ANIT treatment resulted in an ANIT response which did not differ statistically from the combined mestranol–norethynodrel–ANIT effect. The response obtained with Mestranol-ANIT treatment did not differ statistically from the control ANIT effect. Thus, the progestational component of Enovid, norethynodrel, but not mestranol, seems responsible for the potentiation of ANIT at this chosen dose.

We next ran a series of experiments to see whether norethandrolone, Enovid, or acetohexamide potentiated the cholestatic activity of ANIT. The results of this study are shown in Table 5. Norethandrolone and Enovid caused a significant increase in the number of ANIT-treated animals exhibiting cholestasis when compared to the control–ANIT response. Acetohexamide pretreatment caused no significant increase in the cholestatic response of ANIT. Norethandrolone, Enovid, and acetohexamide alone had no effect on bile flow as determined by the fluorescein method.

To determine whether there was an increase in hepatic damage coincidental with

TABLE 5. EFFECT OF NORETHANDROLONE, ENOVID AND ACETOHEXAMIDE ON ANIT-INDUCED CHOLESTASIS

| Treatment* | No. blocked/No. treated† |
|---|---|
| Norethandrolone–ANIT | 10/10‡ |
| Enovid–ANIT | 8/10‡ |
| Acetohexamide–ANIT | 4/10 |
| Control–ANIT | 5/20 |
| Norethandrolone | 0/10 |
| Enovid | 0/10 |
| Acetohexamide | 0/10 |

* Norethandrolone (20 mg/kg, p.o.) or acetohexamide (50 mg/kg, p.o.) was given 24 and 12 hr prior to ANIT (80 mg/kg, p.o.) administration. Enovid (50 mg/kg, p.o.) was given once, 24 hr prior to ANIT.

† The fluorescein test was used to determine the presence or absence of bile flow 10 hr after the administration of ANIT. The number blocked pertains to those showing an absence of bile flow.

‡ Significantly different ($P < 0.05$) from control–ANIT response.

the potentiation of ANIT-induced hyperbilirubinemia and cholestasis, SGPT levels were determined, rather than plasma bilirubin levels or the presence or absence of bile flow. The results of this study are shown in Table 6. Norethandrolone–ANIT treatment resulted in the highest SGPT levels. None of the agents themselves caused an elevation of SGPT.

Figure 1 shows the results of hexobarbital sleeping time and total-body levels of hexobarbital after treatment with norethandrolone, Enovid, and acetohexamide. Norethandrolone and both acute and chronic Enovid treatment significantly shortened hexobarbital sleeping time. Norethandrolone treatment resulted in the greatest shortening of sleeping time, the mean being 33% of the control sleeping time. Acetohexamide on the other hand failed to shorten sleeping time significantly. Total-body levels of hexobarbital were reduced in an identical manner for each of the drug treatments, substantiating an increased rate of hexobarbital metabolism after pretreatment

TABLE 6. SERUM GLUTAMIC-PYRUVIC TRANSAMINASE (SGPT) LEVELS AFTER NORETHANDROLONE, ENOVID AND ACETOHEXAMIDE PRETREATMENT

| Treatment* (N = 10) | SGPT level (units)† | Significance ($P < 0.05$) |
|---|---|---|
| Norethandrolone–ANIT | 230 ± 11 | \| |
| Acetohexamide–ANIT | 114 ± 18 | \| |
| Enovid–ANIT | 101 ± 6 | \| |
| ANIT | 61 ± 14 | \| |
| Control | 21 ± 1 | \| |

* Norethandrolone (20 mg/kg, p.o.) or acetohexamide (50 mg/kg, p.o.) was given 24 and 12 hr prior to ANIT (80 mg/kg, p.o.) administration. Enovid (50 mg/kg, p.o.) was given once 24 hr prior to ANIT administration.

† SGPT levels were determined 24 hr after the administration of ANIT. Control experiments with norethandrolone, acetohexamide, and Enovid alone showed no elevation of SGPT.

with norethandrolone and acute and chronic Enovid, but not with acetohexamide treatment.*

Additional studies of the potentiation of ANIT have been done with three other known cholestatic agents: chlorpropamide, methyltestosterone, and erythromycin estolate. Both methyltestosterone (20 mg/kg) and erythromycin estolate (20 mg/kg) had a very variable effect on ANIT-induced hyperbilirubinemia, and for that reason further studies with these drugs were not conducted. Chlorpropamide (100 mg/kg) on the other hand did significantly potentiate (twofold) ANIT-induced hyperbilirubinemia. However, because of the occurrence of a high mortality rate with the combined ANIT–chlorpropamide treatment, further investigations of this potentiation were not conducted.

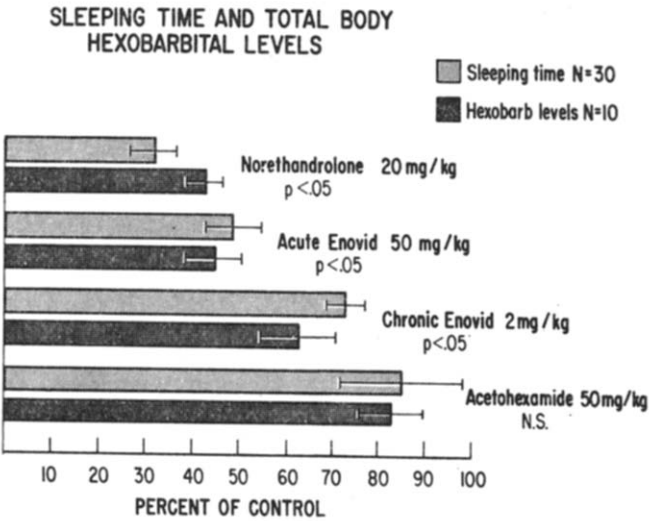


FIG. 1. Norethandrolone (20 mg/kg, p.o.) was administered 24 and 12 hr prior to hexobarbital treatment. Acute Enovid treatment consisted of a single dose (50 mg/kg, p.o.) given 24 hr before hexobarbital administration. Chronic Enovid treatment consisted of five daily doses of 2 mg/kg. Acetohexamide (50 mg/kg, p.o.) was given 24 and 12 hr prior to hexobarbital administration. Sleeping times were determined after hexobarbital, 125 mg/kg, i.p. The mean sleeping time for the contro group was 70 $\pm$ 5 min. Determinations of total-body hexobarbital levels were done 90 min after a dose of hexobarbital, 200 mg/kg, i.p. The mean hexobarbital total-body level for the control group was 75 $\pm$ 5 mg/kg. N equals the number of animals per treatment group. Each bar graph represents a mean value $\pm$ S.E. Each treatment group was compared statistically to the control group by means of the Student's *t* test. The treatments resulting in a significant difference from control values are designated by $P < 0{\cdot}05$, those that were not significant by N.S.

## DISCUSSION

Drug-induced hepatic injury, particularly that associated with jaundice, is becoming more prevalent as indicated by the increasing number of reports appearing in the literature implicating various therapeutic agents with adverse hepatic reactions. The fact that the data presented here show that it is possible to potentiate ANIT-induced

* Juchau and Fouts (personal communication) find that liver microsomes obtained from rats 18–48 hr after treatment with norethynodrel exhibit increased hexobarbital metabolism.

hyperbilirubinemia and cholestasis with certain of the known cholestatic agents lends support to the hypothesis that ANIT may be a useful tool for detecting potential cholestatic agents. Such a technique would be of obvious importance.

The data clearly show that pretreatment with norethandrolone and Enovid results in potentiation of ANIT-induced hyperbilirubinemia and cessation of bile flow in mice. A third drug, acetohexamide, potentiated the hyperbilirubinemic response but did not apparently potentiate the cholestatic response to ANIT. These drugs alone, however, had no apparent effect on bilirubin clearance or bile flow. Norethandrolone and other anabolic steroids have been shown to cause retention of sulfobromophthalein (BSP) in experimental animals after acute administration.[19] We are unaware of any reports showing similar responses with acetohexamide.

Recently, Enovid has been implicated as a cholestatic drug in man.[20, 21] In both instances this response occurred in women with prior histories of cholestatic jaundice associated with pregnancy. In our experiments it is seen that both acute and chronic administration of Enovid results in potentiation of the ANIT responses. From our studies it would appear also that it is the norethynodrel component, rather than mestranol, which is responsible for the potentiation. Recently it has been shown[17] that Enovid causes BSP retention in man and that the apparent BSP-Tm is lowered in these cases. On the basis of indirect evidence these authors also suggested that norethynodrel rather than mestranol causes this effect. Hargreaves[3] reports that norethynordel depresses the excretion of indocyanine green and bilirubin in rats, but the experimental details were not described in the report.

In our experimental situation chlorpropamide significantly potentiated ANIT-induced hyperbilirubinemia, and pretreatment with erythromycin estolate or methyltestosterone resulted in very variable responses to ANIT. All three of these drugs have produced jaundice in man.[1] Since only acute pretreatments were tried with these drugs, we do not know whether potentiation of ANIT might occur under different experimental conditions.

However, certain of the results presented in this paper indicate that the potentiation of ANIT may not necessarily be restricted specifically to agents which have the potential of causing hepatic cholestasis. The hexobarbital studies in particular draw attention to the possibility that the potentiation of ANIT is closely related to the degree of activity of the hepatic microsomes. Our previous work with chlorpromazine and phenobarbital[8] showed that their ANIT-potentiating properties correlated well with their stimulatory effects on microsomal metabolism. Also, it was possible to induce potentiation of ANIT during the microsomal stimulatory phase of SKF 525-A (β-diethylaminoethyldiphenylpropylacetate HCl).[8] The potentiation of ANIT effects therefore may be due to an increased rate of microsomal metabolism resulting in the conversion of ANIT to an active metabolite which is responsible for the hyperbilirubinemia and cholestasis. If this were true the specificity of ANIT potentiation would not be restricted to compounds that have cholestatic potential but to compounds that stimulate the critical pathways of ANIT metabolism. However, nothing is known about ANIT metabolism, let alone whether a biotransformation product is responsible for its action.

It is quite striking that many of the drugs which have been associated with cholestasis in man are also known to stimulate certain hepatic microsomal enzyme systems (norethandrolone, phenylbutazone, methyltestosterone, chlorpromazine, tolbutamide,

and urethan).[22–24] Jaundice has also been reported with chronic phenobarbital treatment.[25] Therefore, an alternative explanation might be that ANIT and/or its metabolite affects those enzymes involved in bilirubin secretion into bile and that these microsomal-stimulating agents also affect this pathway. ANIT has been found to inhibit several microsomal pathways both *in vivo* and *in vitro*.[26] It is not unreasonable, therefore, to suppose that ANIT also affects a bilirubin secretion transport system. Since little is known regarding the possible enzymic characteristics of a bilirbuin transport system, means of testing the hypothesis are not clear. We recognize that the hepatic injury associated with some of these drugs in man occurs with a low frequency and is sometimes accompanied by manifestations of hypersensitivity. It is possible, therefore, that actually only a few of these agents exert a direct hepatotoxic effect, while others elicit their reactions through hypersensitivity.

It is our judgement that until the mechanisms involved in the potentiation are elucidated, definite conclusions cannot be made regarding the usefulness of ANIT in determining the presence or absence of cholestatic activity in drugs. However, in view of the overall problem of drug-induced cholestasis in man, every effort should be made to clarify this experimental situation.

*Acknowledgements*—The authors wish to express appreciation to Dr. V. A. Drill, G. D. Searle & Co., for supplying norethandrolone, Enovid, mestranol, and norethynodrel; to Dr. N. Belcher, Chas Pfizer & Co., for supplying chlorpropamide; and to Dr. J. M. McGuire, Eli Lilly & Co., for supplying acetohexamide and erythromycin estolate.

## REFERENCES

1. H. Popper, E. Rubin, D. Gardiol, F. Schaffner and F. Paronetto, *Arch. intern. Med.* **115**, 128 (1965).
2. V. A. Drill, *Ann. N.Y. Acad. Sci.* **104**, 858 (1963).
3. T. Hargreave;, *Nature, Lond.* **206**, 154 (1965).
4. S. Sherlock, *Ann. Rev. Pharmac.* **5**, (1965).
5. B. A. Becker and G. L. Plaa, *Toxicol. appl. Pharmac.* **7**, 708 (1965).
6. S. Goldfarb, E. J. Singer and H. Popper, *Am. J. Path.* **40**, 685 (1962).
7. H. Ungar, E. Moran, M. Eisner and M. Eliakim, *Archs Path.* **73**, 427 (1962).
8. R. J. Roberts and G. L. Plaa, *J. Pharmacol exp. Ther.* **150**, 499 (1965).
9. P. V. Ferro and A. B. Ham, *Am. J. Clin. Path.* **40**, 209 (1963).
10. G. L. Plaa and B. A. Becker, *J. appl. Physiol.* **20**, 534 (1965).
11. J. Axelrod, J. Reichenthal and B. B. Brodie, *J. Pharmac. exp. Ther.* **112**, 49 (1954).
12. S. Reitman and S. Frankel, *Am. J. clin. Path.* **28**, 56 (1957).
13. R. G. Steel and J. H. Torrie, *Principles and Procedures of Statistics*. McGraw Hill, New York (1960).
14. F. Schaffner, H. Popper and E. Chesrow, *Am. J. Med.* **26**, 249 (1959).
15. Council on Drugs, *J. Am. med. Ass.* **191**, 127 (1965).
16. N. E. Borglin, *Br. med. J.* **1**, 1289 (1965).
17. G. J. Kleiner, L. Kresch and I. M. Arias, *New Engl. J. Med.* **273**, 420 (1965).
18. U. Larsson-Cohn and U. Stenram, *J. Am. med. Ass.* **193**, 422 (1965).
19. H. D. Lennon, *Steroids* **5**, 361 (1965).
20. A. J. Elliott and J. Hendry, *Canad. med. Ass. J.* **92**, 334 (1965).
21. W. C. Boake, S. G. Schade, J. F. Morrissey and F. Schaffner, *Ann. intern. Med.* **63**, 302 (1965).
22. C. L. Rümke and J. Bout, *Naunyn-Schmiedeberg's Archs exp. Path. Pharmak.* **240**, 218 (1960).
23. A. H. Conney and K. Schneidman, *J. Pharmac. exp. Ther.* **146**, 225 (1964).
24. J. Booth and J. R. Gillette, *J. Pharmac. exp. Ther.* **137**, 374 (1962).
25. D. G. Welton, *J. Am. med. Ass.* **143**, 232 (1950).
26. G. L. Plaa, L. A. Rogers and J. R. Fouts, *Proc. Soc. exp. Biol. (N.Y.).* **119**, 1045 (1965).